Lipid Oxidation and its Relationship to Lipid Bilayer Permeability in the Oyster *Crassostrea virginica*

An Honors Thesis (HONRS 499)

By

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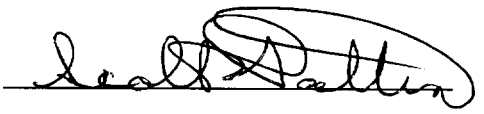

Ball State University

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This rough draft of an article to be published in *Comparative Physiology and Biochemistry* is the culmination of an honors college fellowship and thesis. In the spring of 1997, I approached Dr. Scott Pattison about beginning a research project. Soon after a project had been defined I applied for and received an honors college fellowship for summer 1997, fall 1997, and spring 1998. I began work on the project in May of 1997. The project involved using biochemical methods to investigate zinc transport across lipid membranes. Dr. Pattison has been researching zinc transport for fifteen years using in vitro methods. His current hypothesis is that oxidized lipids help to form pores that facilitate zinc transport. Some of the groups on the research team are doing kinetics studies using lipids from eggs, which have varying degrees of oxidation, to study how long it takes zinc to enter an artificial membrane.

I have a good biology background, so I proposed that we utilize an animal model to study the transport. After some research and much discussion, we decided to use oysters in our project. The next task was to find out how to get oysters and how to care for them once they arrived. The local grocery stores did not carry live oysters, so we checked with local restaurants. Vann's restaurant near the airport gets shipments of oysters weekly, so we put in an order with their supplier. Soon we had a dozen oysters in a 20-gallon fish tank living in salt water.

A battery of tests was developed to give the data needed to further the zinc transport project. Each day of the summer session I would process one or

two oysters, as time would allow. Processing one oyster could take up to six hours. First the oyster would have to be prepared for biochemical analysis. The digestive gland was dissected out, weighed, and homogenized in water. The first test performed on the gland homogenate measured the amount of malonaldehyde, which is a byproduct of oxidized lipid. The next test, called the Bradford assay measures the amount of protein in the homogenate. Knowing how much protein is in the gland is important because it allows the other data to be normalized. All other data can be compared to the amount of protein in the cell. A third test quantified the zinc in the sample using atomic absorption techniques. The final test tells about the metabolic activity of the animal by measuring the activity of a common nerve impulse transmitter.

The goal of the experiment was to see if oysters with high levels of malonaldehyde also have high levels of zinc. In fact, this is what we did find. The results and conclusions in the article thoroughly address the findings and implications of this research project.

This project was very important to my academic career. Many of the graduate schools that I interviewed with do not offer interviews to candidates without research experience. Also, this project allowed me to make a smooth transition from chemistry undergraduate work to a biological graduate school appointment. This experience of developing a project and seeing it through to publication is excellent preparation for graduate school where thesis and research publications are the primary goals. Additionally, the techniques used in the tests utilized many different instruments including the flurometer, the

UV/vis spectrometer, and the atomic absorption spectrometer. Knowing how to use instruments, which are essential to biochemical research will be a great asset to my research career.

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Introduction

Zinc is an essential trace metal, which has been extensively studied (1). Transport mechanisms for all more abundant metals have been found, but the mechanism of zinc transport is still unclear. Since zinc is found and transported in relatively small amounts, it is difficult to study. Rat hepatocyte research has resulted in many proposed mechanisms, which include active transport, facilitated and/or passive diffusion (2,3). Model studies using protein-free liposomes shows transport rates similar to those found in rat hepatocyte studies (4).

This paper examines zinc transport in a model organism, *Crassostrea Virginica*. Mollusks accumulate environmental pollutants, including metals (5). Oysters accumulate higher concentrations of metals than other mollusks (6). The concepts of accumulation and excretion are based on the amount of zinc, which enters or leaves a cell. For approximately one hundred years, nutrient metabolism has been graphically represented using the following equation (7). Total substance = constant * body wt.^{b}. When a substance is not excreted or accumulated, $b=1$ showing that the substance equilibrates between the environment and the growing organism. Any $b<1$ suggests that some force is preventing the substance from entering the cell freely. Also, the graph shows that as the oyster grows the concentration of the substance decreases. Any $b>1$ indicates accumulation or inability to excrete. A graph of this function indicates as the oyster grows the concentration of the substance increases.

The digestive glands of oysters are assayed to determine the degree of oxidation of lipid(malonaldehyde concentration,[MDA]), zinc content, metabolic activity(AChE), and protein concentration. A linear relationship was found when [MDA] was graphed vs. [Zn]. This trend leads us to conclude that lipid oxidation facilitates zinc diffusion across lipid membranes.

Materials and Methods

Oysters were supplied by Midwest Seafood Supply and kept in seawater (supplied by Instant Ocean Inc.) at approximately 20° C. All tests were performed on the day digestive gland was removed. The gland was homogenized in 10 weight equivalents of water.

Determination of total MDA

The thiobarbituric acid test was used as described in the literature (3). A Spex Fluorolog flourimeter was used.

Determination of [protein]

The Bradford assay was used as described by Sigma. A Hewlett Packard 8452A Diode Array Spectrophotometer was used.

Determination of AChE

AChE specific activity was determined using the Ellman assay (7). A Hewlett Packard 8452A Diode Array Spectrophotometer was used.

Determination of total zinc

Oyster homogenate was diluted to fit into the analytical range of the Perkin Elmer Zeeman Atomic Absorption Spectrometer.

Results

Control of metal accumulation.

Plotting total metal vs. dry body weight yields an exponential function (Total metal = constant * body wt.^{b}) (fig. 1). This equation is based on the idea that bodily functions change with body size. Two common aging trends should be recognized. First, metabolic rate decreases with size and secondly, feeding rate decreases to a greater degree. As the equation indicates, this is an exponential relationship. The magnitude of this exponent tells indirectly how the rate of metal metabolism is related to bodily functions. When water filtration is plotted vs. body weight, $b \approx 0.4$. If total metal is plotted vs. body weight and a "b" value is found similar to the one for filtration vs. body weight, there is a correlation between total metal and filtration. Exponents of 0.75 link metal intake to metabolism. When metal enters and leaves passively $b = 1$ and $b > 1$ shows metal accumulation. Metal accumulation occurs when a means of storage is developed to protect the cell in the absence of an excretion method. Metabolic relationships of this type are easier to recognize when total metal vs. dry body weight is graphed on log-log scales so that the slope of the line reveals the exponent.

Dry weight gives a more accurate estimate of oyster size than gland weight or shell size. Plotting experimental total Zn (ug) vs. digestive gland weight (g) yields $b = 1.21$ (fig. 2) where total Zn vs. dry wt. yields $b = 1.32$ (fig. 3). Linearity of the data is also greater when dry wt. is used compared to the use of gland weight. Digestive gland zinc concentration (ug Zn/mg protein) vs. dry wt. is linear with a positive slope showing that larger oysters have accumulated more zinc than smaller ones (fig. 4).

Total oyster MDA vs. gland weight gives $b = 0.75$ linking it to energy metabolism (fig. 5). An exponent of less than one means larger oysters have a smaller concentration of MDA than smaller oysters. [MDA] may be smaller for several reasons. Slower metabolism produces fewer oxidizing byproducts causing less damage. Also, older oysters might accumulate antioxidant scavengers providing additional protection against oxidation. Larger oysters also have proportionately less exposed surface area in the gland further protecting them.

Total AChE vs. gland weight shows a strong correlation where larger glands possess more AChE (fig. 6). This plot also shows a relationship to

METAL ACCUMULATION AND SHELLFISH METABOLISM

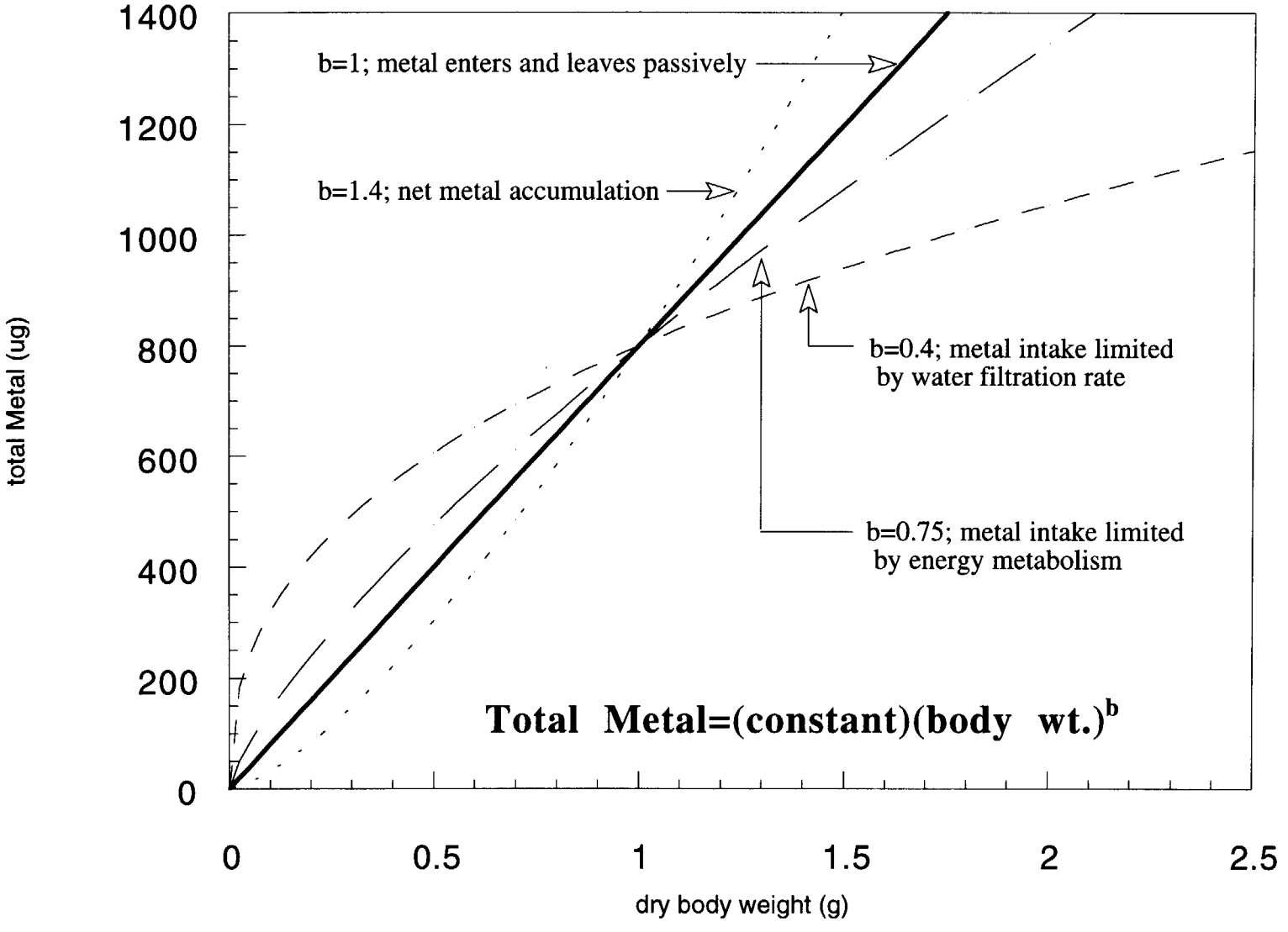

FIGURE 1

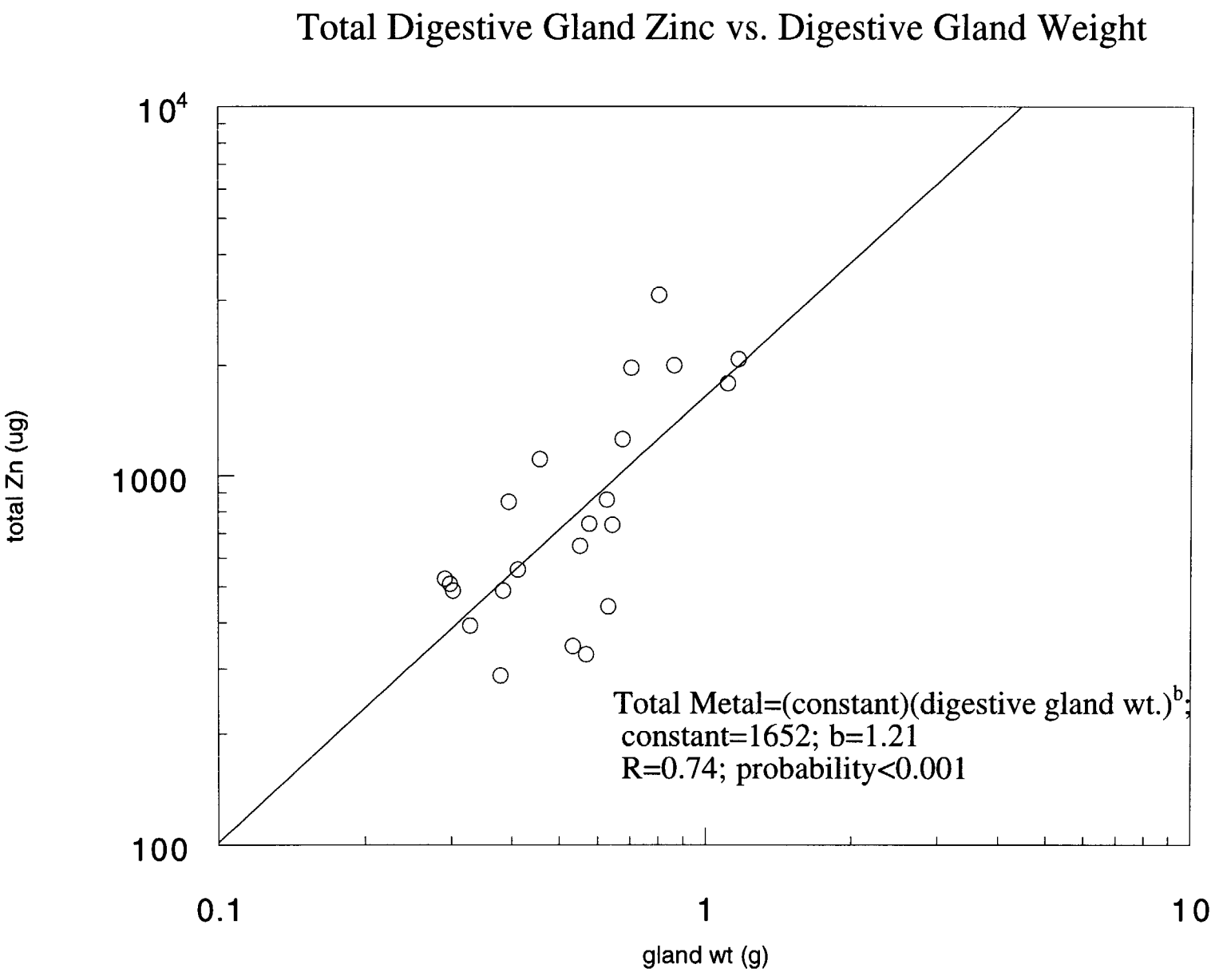
Total Digestive Gland Zinc vs. Digestive Gland Weight
10^4
1000
100
total Zn (ug)
0.1
1
10
gland wt (g)
Total Metal=(constant)(digestive gland wt.)^b;
constant=1652; b=1.21
R=0.74; probability<0.001

FIGURE 2

Total Digestive Gland Zinc vs. Oyster Dry Weight

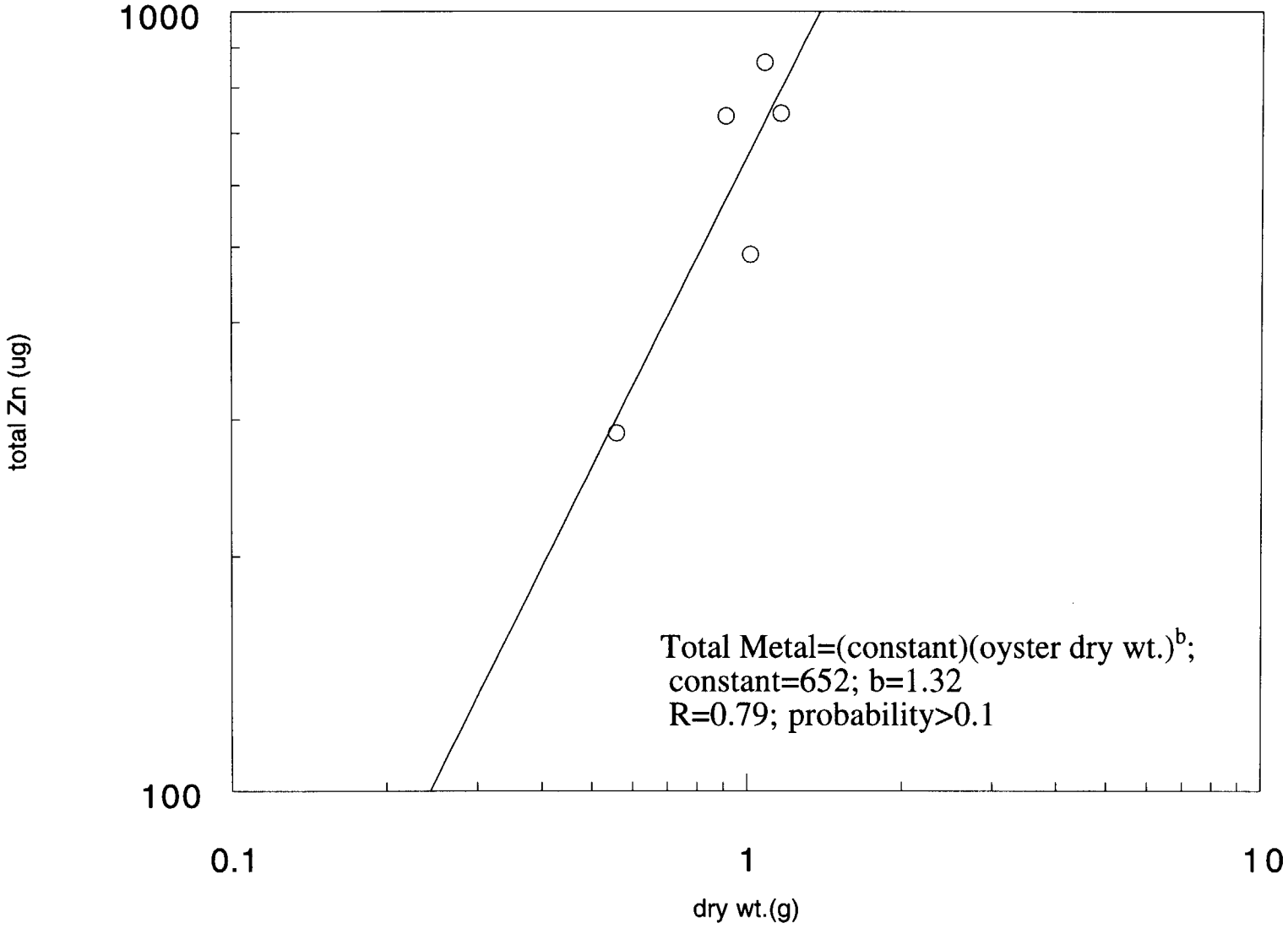

FIGURE 3

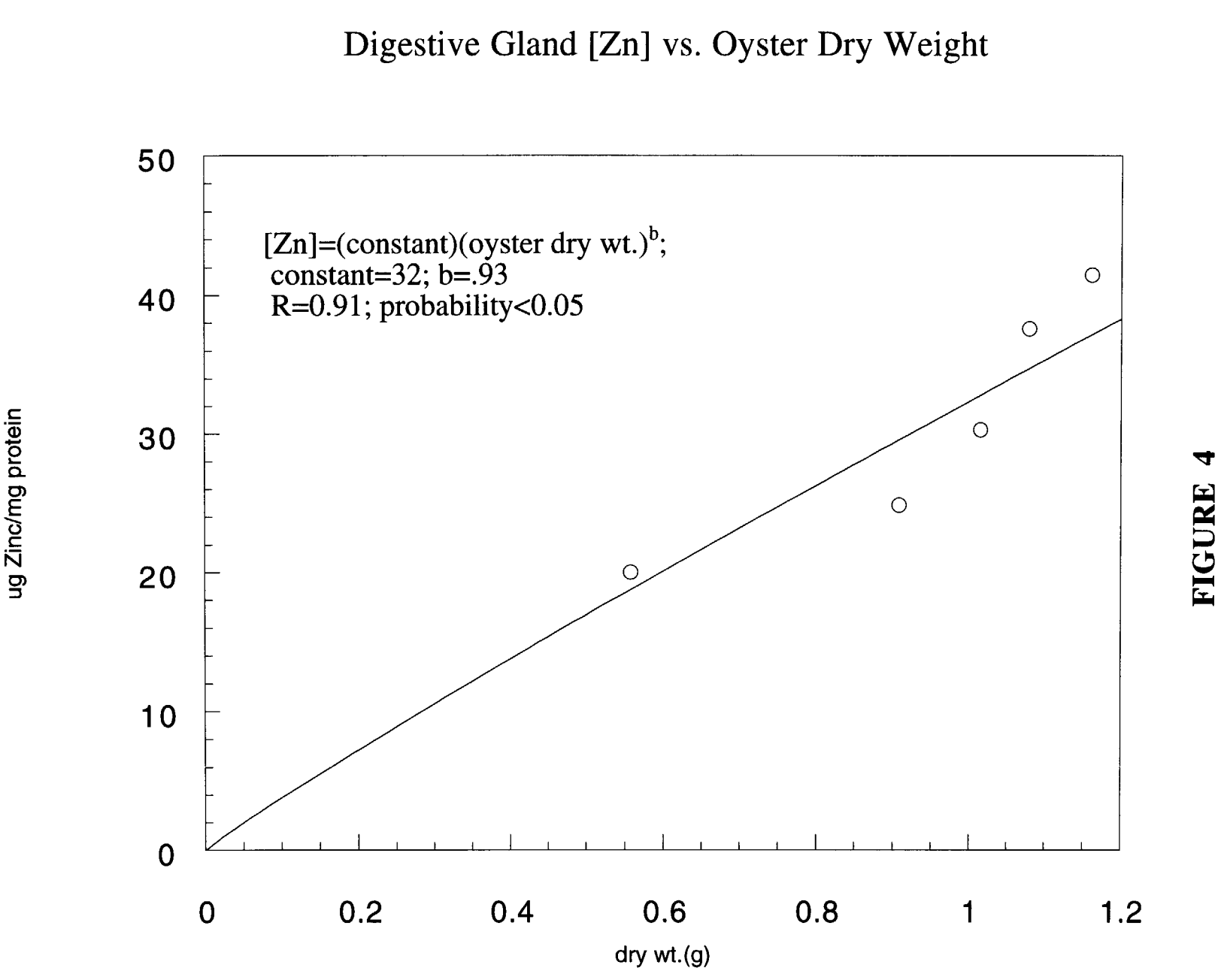
Digestive Gland [Zn] vs. Oyster Dry Weight
[Zn]=(constant)(oyster dry wt.)^b;
constant=32; b=.93
R=0.91; probability<0.05
ug Zinc/mg protein
dry wt.(g)
0
10
20
30
40
50
0
0.2
0.4
0.6
0.8
1
1.2

FIGURE 4

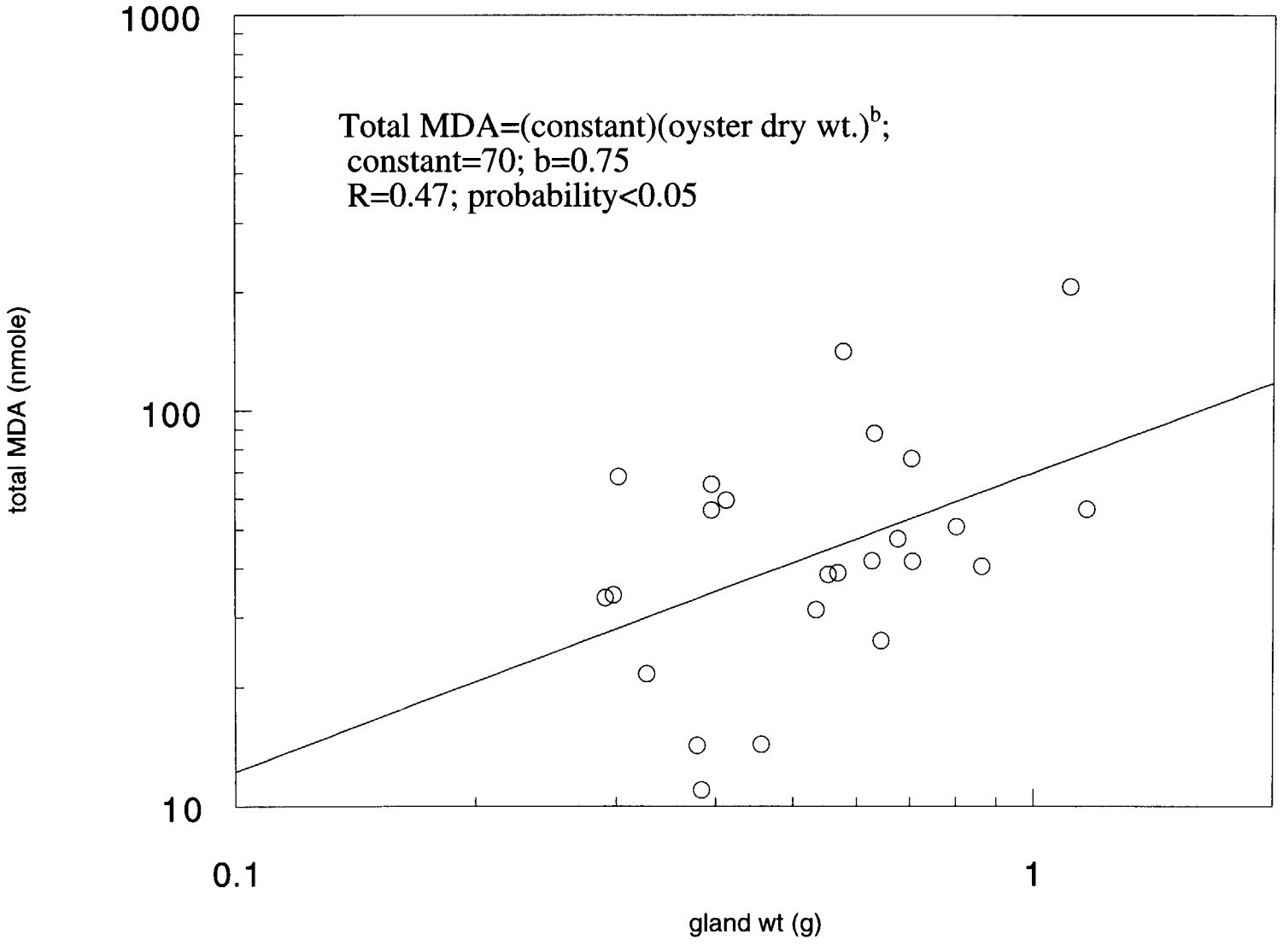
Total Lipid Oxidation vs. Digestive Gland Weight
Total MDA=(constant)(oyster dry wt.)^b;
constant=70; b=0.75
R=0.47; probability<0.05
1000
100
10
0.1
1
total MDA (nmole)
gland wt (g)

FIGURE 5

Total Acetylcholinesterase Activity vs. Digestive Gland Weight

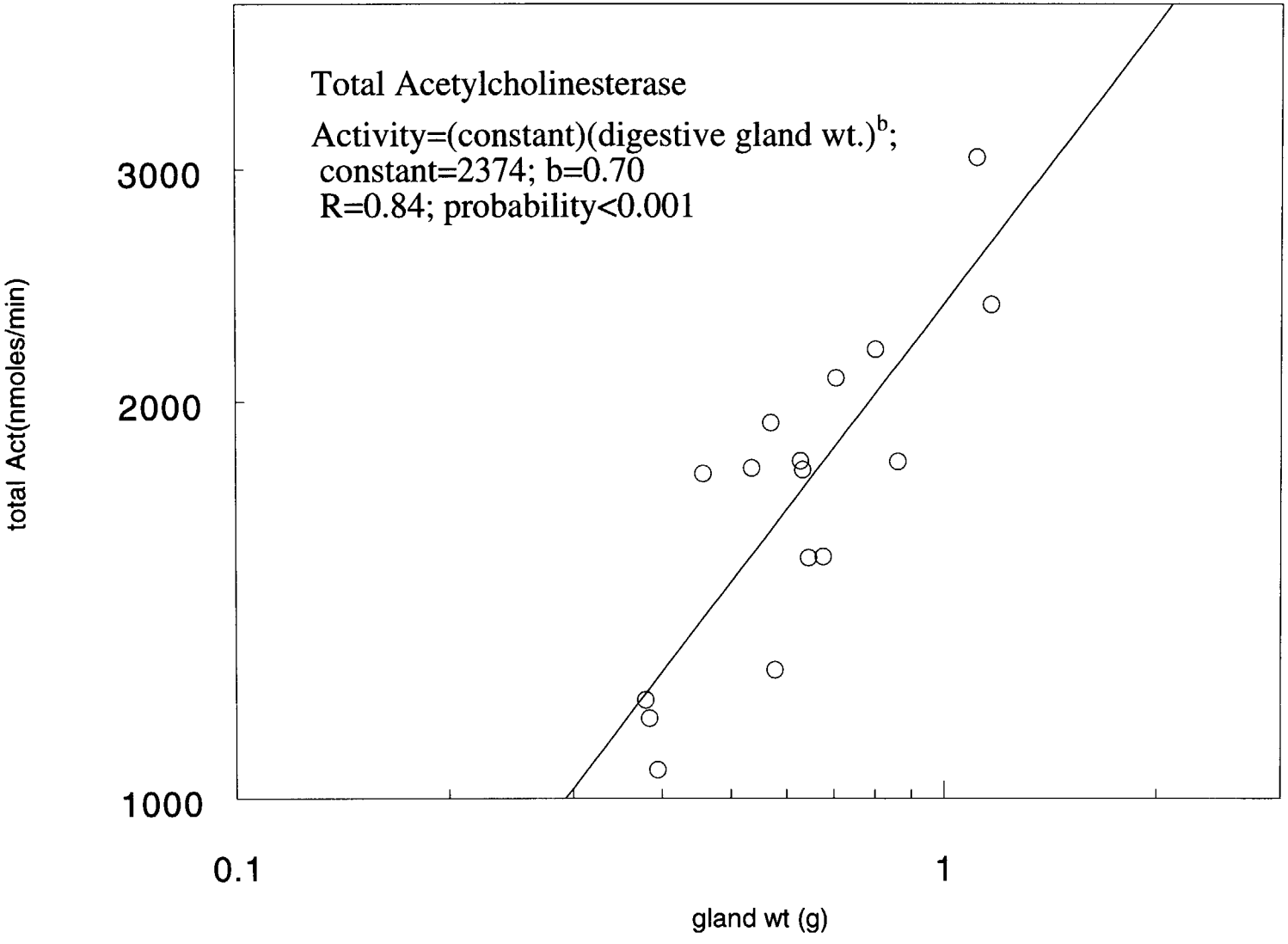

FIGURE 6

energy metabolism with b=0.70. Another graph showing MDA concentration vs. AChE specific activity displays the relationship between high oxidation and high-energy metabolism (fig. 7).

The Zn concentration vs. MDA concentration plot reveals a correlation between lipid oxidation and Zn concentration (fig. 8). The Zn/MDA vs. tank Zn concentration plot indicates that the amount of Zn found per oxidized lipid depends linearly on the amount of Zn in the environment (fig. 9). Accordingly oysters in an environment of higher zinc will accumulate more zinc than those in a low zinc environment while having identical MDA levels.

Discussion

As shown in results, Zinc is accumulated in the digestive glands of oysters (see fig. 3). Higher concentrations of Zn were found in the digestive gland than in the surrounding water. This was expected based on literature reports and the fact that zinc "sticks" to proteins. Zinc's affinity for protein keeps zinc inside the cell once it enters because the interior of a cell is more protein rich than the outside, thus zinc transport is essentially one-way.

More total zinc was found in larger digestive glands, which is expected because there are more cells. Additionally, the concentration of zinc was also higher in larger glands. This is demonstrated by the exponent found from oyster data and the equation included in the Introduction. The equation (Total metal = constant * body wt.^{b}) yields an experimental exponent of 1.32, which is greater than one indicating accumulation. Any exponent greater than one means that some factor is keeping the zinc from leaving the cell. In the same way, an exponent less than one means that there is some factor keeping zinc from entering the cell. Also, exponents less than one indicate higher concentrations in smaller oysters, while exponents greater than one indicate higher concentrations in bigger oysters. From this data, it might be concluded that as the oyster grows, MDA levels approach a maximum value, but zinc may increase only limited by environmental supply.

As indicated by the equation in the Introduction, total MDA and total AChE vary with energy metabolism. A plot of MDA vs. gland weight gave the exponent which indicates a relationship to energy metabolism, 0.75. Since the exponent is less than one, this means that smaller oysters have a higher concentration of MDA. This is counter to findings in other studies where older (bigger) bivalve molluscs are more susceptible to oxidative damage. These results may be explained many ways. First, the smaller oysters may not have sufficiently developed oxidative defenses. This would cause them to be more vulnerable to oxidative attack. It is also possible that older oysters have had the opportunity to accumulate antioxidant scavengers from their food supply. Another explanation involves the surface area of small glands as compared to larger glands. Smaller glands have unproportionately larger surface area than larger glands. This means that smaller glands are more exposed to any oxidant.

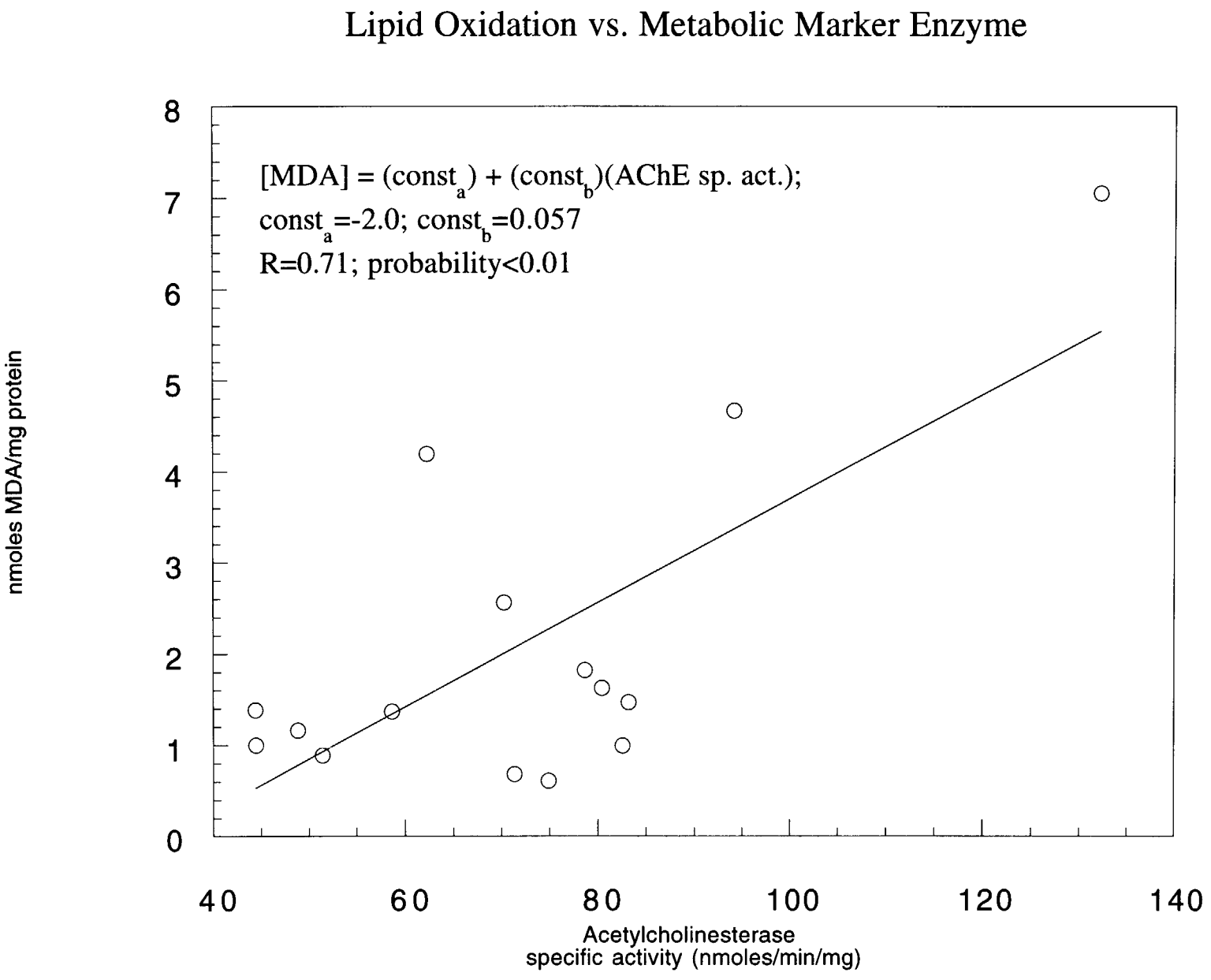
Lipid Oxidation vs. Metabolic Marker Enzyme
[MDA] = (const$_a$) + (const$_b$)(AChE sp. act.);
const$_a$=-2.0; const$_b$=0.057
R=0.71; probability<0.01
nmoles MDA/mg protein
0
1
2
3
4
5
6
7
8
40
60
80
100
120
140
Acetylcholinesterase
specific activity (nmoles/min/mg)

FIGURE 7

Digestive Gland
[Zn] vs. Lipid Oxidation, [MDA]

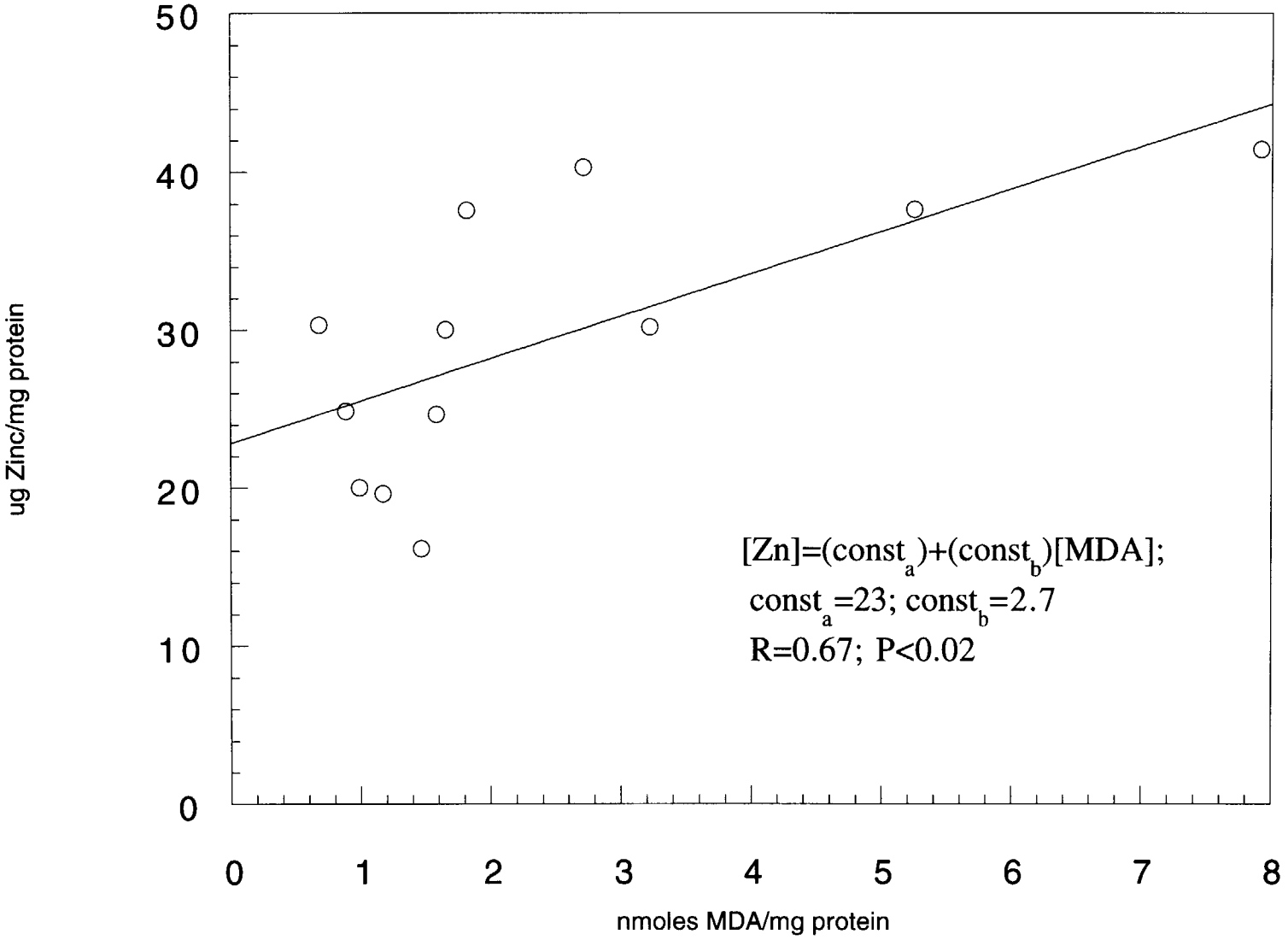

FIGURE 8

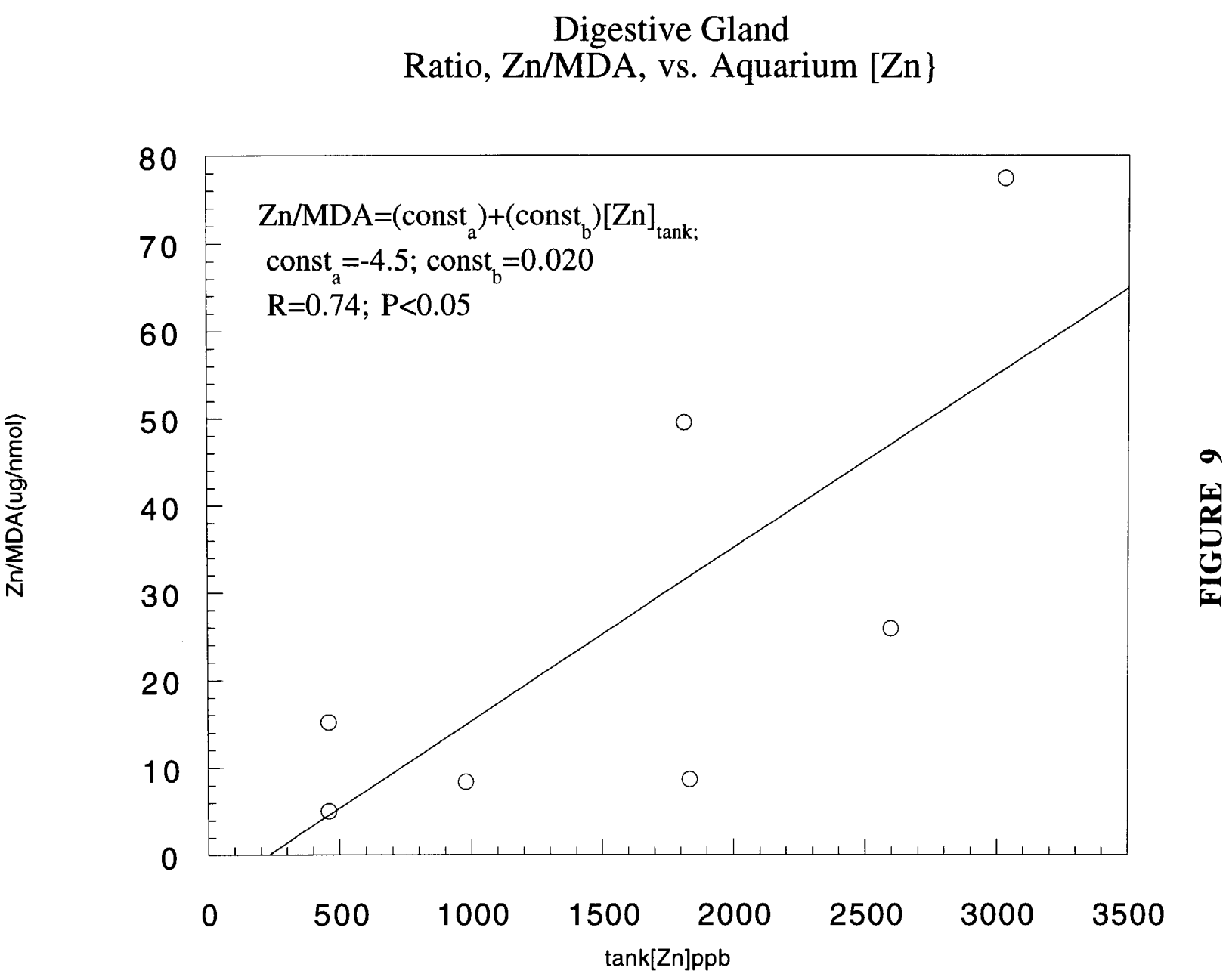
Digestive Gland
Ratio, Zn/MDA, vs. Aquarium [Zn}
Zn/MDA=(const_a)+(const_b)[Zn]_tank;
const_a=-4.5; const_b=0.020
R=0.74; P<0.05
Zn/MDA(ug/nmol)
tank[Zn]ppb
0
10
20
30
40
50
60
70
80
0
500
1000
1500
2000
2500
3000
3500

FIGURE 9

Total AChE vs. gland weight also yielded an exponent showing connection to energy metabolism, 0.7. The correlation of AChE with energy metabolism was expected because of the nature of oyster feeding and digestion. The primary reason for oyster movement is water filtration for the purposes of obtaining nutrients. So, any significant movement should be directly related to energy metabolism. Since the exponents from the MDA and AChE plots are similar, the correlation between [MDA] and AChE specific activity can be predicted.

Similar exponents link metabolic activity with oxidative damage. Many physiological factors predict this relationship. It is not clear whether this damage is due to exposure to the environment involved with feeding or if cellular defenses against oxidative digestive byproducts are not adequate. Oyster feeding requires that the oyster be open and actively pumping water through its gills. This close relationship with the environment makes the oyster especially vulnerable to oxidizing agents commonly found in oceans. These include metals, which undergo reduction and oxidation like Fe and Cu and halogenated organic chemicals such as CCl_4 or $CHCl_3$. Inadequate cellular defenses may also allow the oyster to damage itself. During metabolism in mitochondrion, oxidizing species escape and cause cellular damage possibly to the lipid membranes. During times of greater metabolic activity, more oxidizing agents are released into the cell. During the summer when metabolic activity peaks, MDA levels are average possibly because of a dietary intake of antioxidants (8). Oysters also undergo seasonal changes in physiology in response to life cycle as well as environmental change (9). During the winter months oysters are less metabolically active due to lower temperatures and lower food availability. Related molluscs have been shown to have lower concentrations of scavenging anti-oxidant species during these months (10). Low concentrations of these scavengers such as glutathione, vitamin E and antioxidant enzymes result in higher MDA values. This lack of scavengers may be due to a low intake of antioxidants, which may be found in the food. So even though metabolic activity is low during the winter months, MDA levels are high due to low dietary intake of antioxidants.

As shown in the results, MDA concentration and zinc concentration are positively correlated. Since both zinc and MDA concentrations vary in different ways with body weight, the weight range of the oysters was limited. Only oysters with gland weights between 0.39g and 0.1g were used. Oysters with high MDA concentrations also have high zinc concentrations. When lipids are oxidized they become slightly more polar and are attracted to other oxidized lipids by hydrophilic interaction. A slightly more polar area in the membrane would be more permeable to charged particles like metal ions than an unoxidized area. The greater the degree of oxidation the larger the number of areas where metals like zinc might enter the cell.

In summary, oysters sequester Zn as they grow, in the absence of an excretion method. Larger oysters are less sensitive to oxidative damage. Larger oysters have lower AChE concentrations, which coincides with lower

metabolic rates. Most importantly, this paper links lipid oxidation to Zn accumulation. Oxidized lipid "pores" provide places for polar species such as Zn^{+2} to cross lipid membranes. Since Zn binds to sites inside the cell this transport is one-way and results in accumulation.

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